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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/31, C12P 7/06
A23C 19/00

A1

(11) International Publication Number: WO 91/19802

(43) International Publication Date: 26 December 1991 (26.12.91)

(21) International Application Number: PCT/EP91/01109

(22) International Filing Date: 12 June 1991 (12.06.91)

(30) Priority data:

9013577.3	18 June 1990 (18.06.90)	GB
9023380.0	26 October 1990 (26.10.90)	GB

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MG, NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: NOVEL BACTERIOCIN FROM A STRAIN OF LACTOCOCCUS LACTIS SUBSP. CREMORIS

1-Lys Leu Thr Phe Ile Gln Ser Thr Ala Ala Gly Asp Leu Tyr Tyr

(I)

16-Asn Thr Asn Thr His Lys Tyr Val Tyr Gln Gln Thr Gln Asn Ala

31-Phe Gly Ala Ala Ala Asn Thr Ile Val Asn Gly Trp Met Gly Gly

46-Ala Ala Gly Gly Phe Gly Leu His His

Glu Lys Asp Ile Ser Gln Glu Glu Arg Asn Ala Lai Asn Ile Ala Glu

Lys Ala Lai Asp Asn Ser Glu Tyr Lai Pro Lys Ile Ile Leu Asn Leu Arg Lip Ala Leu

Thr Pro Leu Ala Ile Asn Arg Thr Leu Asn Ths Asp Leu Ser Glu Leu Tyr Lys Phe Ile

(II)

Thr Ser Ser Lys Ala Ser Ans Lys Asn Leu Gly Gly Gly Lei Ile Met Ser Trp Gly Arg Leu Phe

(57) Abstract

The invention provides a polypeptide having or including the amino acid sequence (I) and derivatives and fragments thereof having bacteriocin activity and a polypeptide having or including the amino acid sequence (II) and derivatives and fragments thereof having bacteriocin immunity activity.

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Novel bacteriocin from a strain of Lactococcus lactis subsp. cremoris

This invention relates to a novel bacteriocin and its isolation, synthesis and use.

We have isolated a novel bacteriocin from a strain of Lactococcus lactis subsp. cremoris. This strain has never been described in the literature.

The above microorganism is autolytic when cell concentrations are high and we have discovered that it produces a bacteriocin which is found extracellularly in growth media, for example M17 medium. The autolysis of the bacteria can be shown to be due to the lytic properties of the bacteriocin. Lysis is prevented by the addition of proteases that degrade the bacteriocin.

Term "bacteriocin" is used herein to include substances released by bacteria which kill not only the productive organism itself, but also other strains of bacteria, by any mechanism, including lysis. Thus, the new bacteriocin here concerned has been shown to inhibit the growth of more than 120 strains of lactococci, including strains producing the known bacteriocins diplococcin and nisin. However, the productive organism itself carries a gene coding for an immunity factor providing resistance to the bacteriocin to prevent indiscriminate lysis and the bacteriocin only seems to lyse its productive organism at high cell concentrations.

We have determined the amino acid sequence of the new bacteriocin. According to one aspect of the present invention, we provide a polypeptide having or including the amino acid sequence:

1-Lys Leu Thr Phe Ile Gln Ser Thr Ala Ala Gly Asp Leu Tyr Tyr

16-Asn Thr Asn Thr His Lys Tyr Val Tyr Gln Gln Thr Gln Asn Ala

31-Phe Gly Ala Ala Ala Asn Thr Ile Val Asn Gly Trp Met Gly Gly

46-Ala Ala Gly Gly Phe Gly Leu His His

and derivatives and fragments thereof having bacteriocin activity.

The above structure is different from that of nisin and has no meaningful sequence homology with other known polypeptide sequences in the SWISS PROT data bank. The lytic activity of the bacteriocin of the invention is substantially greater than that of previously known L.lactis bacteriocins.

The novel bacteriocin is heat stable and retains activity after boiling in water for 30 minutes. This is of value in permitting its use in industrial processes using L.lactis organisms for example cheese and yoghurt manufacture.

The bacteriocin appears to kill lactococci by lysis. Accelerated lysis of lactococci is beneficial in accelerating cheese ripening and the new bacteriocin is thus of particular application in the production of cheese.

The lytic activity of the bacteriocin may also be of use in production of cell wall preparations or for liberation of nucleic acid material.

Since certain bacteria, for example Gram-negative bacteria, are resistant to bacteriocins, negative selection is possible by using the bacteriocin according to the invention to remove certain cells, for example L.lactis, from mixed cell populations e.g. in starter cultures for fermentation.

Where the productive strain of L.lactis is used as the sole or principle organism in an industrial process such as cheese or yoghurt production, addition of the bacteriocin of the invention to the starter culture serves to eliminate foreign organisms and may be effective against, for example, spore forming clostridia or unwanted strains of L.lactis.

The bacteriocin may advantageously be added to a cheese or yoghurt fermentation at a relatively late stage, after lactic acid, protease and flavour production by the L.Lactis organism has already taken place.

By keeping the productive strain pure, either in the starter culture or in the milk or other medium, unformity of production can be improved.

The bacteriocin may also be used to kill selectively strains of lactic acid producing bacteria in beer and distillery fermentations, since these are attributed in the literature to be the major causes of spoilage in unpasteurised beers and give rise to the greatest proportion of infections during fermentation.

The invention particularly includes starter cultures of microorganisms containing the bacteriocin as an inhibitor of contaminating lactococcus species. Such microorganisms may, for example, be strains of L.lactis resistant to the bacteriocin e.g. the producing organism, so that only unwanted microorganisms are removed from the starter culture, or yeasts of use in beer or distillery fermentations. Such starter cultures will normally be in lyophilised form.

In view of its high specificity, the bacteriocin may be used as a taxonomic tool in the identification of Lactococcus species.

The new bacteriocin may be isolated from cultures of Lactococcus lactis subsp. cremoris by fractionation of the growth medium whereby fractions enriched in the bacteriocin are collected. By applying known fractionation techniques it is possible to obtain the bacteriocin in electrophoretic purity. Thus for example, the organism may be grown in a suitable culture medium, e.g. M17 broth, and the supernatant subjected to

fractional precipitation e.g. with ammonium sulphate, followed by chromatography e.g. on carboxymethyl agarose with elution with phosphate buffer and/or on phenylsuperose with gradient elution with phosphate buffer containing increasing concentrations of ethanol.

We have been able to clone and sequence a two gene operon from L. lactis cremoris which codes for the bacteriocin in the pro-form as well as a further protein which is believed to be the immunity factor providing resistance against self-destruction by the bacteriocin. The full sequence of the operon is shown in Fig. 1 which also shows the sequence of the corresponding proteins, i.e. the bacteriocin and the immunity factor. The operon starts with a regulating region for expression of the genes. This is believed to comprise promoters at regions -35 and -10 and a ribosomal binding site. The gene coding for the pro-sequence runs from base 312 to base 374. The gene coding for the bacteriocin runs from base 375 to base 536. The gene coding for the putative immunity factor runs from base 554 to base 847 in a different reading frame. Three putative promoter sequences are indicated as P1, P2 and P3 in regions -35 and -10 and ribosome binding sites are indicated as RBS.

According to a further feature of the invention we provide DNA coding for the bacteriocin and for the immunity factor respectively. It will be appreciated that knowledge of the overall amino acid sequence shown in claim 1 and/or the DNA sequence coding for the pro-bacteriocin does not provide an indication of the position of the first codon coding for the mature bacteriocin.

The invention thus includes not only the DNA sequences shown in Fig. 1 but also sequences which due to the degeneracy of the code, are also capable of coding for

the proteins concerned.

The invention also includes cloning and expression vectors containing the DNA coding for the mature bacteriocin and/or for the immunity factor. Expression vectors appropriate to L. lactis are particularly preferred.

The invention also includes strains of L. lactis transformed with such vectors.

The immunity factor according to the invention may be of use in combating the effects of L. lactis bacteriocins, for example, in controlling the effects of the bacteriocin according to the invention.

The gene coding for the immunity factor may be used as a selective marker in future construction of food grade cloning vector, for example instead of an antibiotic matter.

The operon shown in Fig. 1 was obtained from the fragmented plasmid DNA of L. lactis cremoris, using a probe comprising all or part of the non-coding DNA strand corresponding to the mature bacteriocin coding portion of the DNA strand shown in Fig. 1. The DNA coding for the mature bacteriocin or immunity factor may be incorporated into any convenient cloning vector for amplification and into an expression vector for transformation of host microorganisms such as L. lactis, for example cloning vector pIL253 (A. Chopin, Biochimie 70, 1988, 59-566). Growth under suitable culture conditions will provide the bacteriocin in the growth medium, from which it can be isolated by the techniques described above.

Furthermore, strains of L. lactis may be transformed

with multiple copies of a plasmid or other vector containing the required DNA sequence to provide an improved strain giving rise to enhanced production of the bacteriocin. Such improved strains may provide more rapid lysis and hence accelerated cheese ripening when used in cheese manufacture. In particular, the strain of L.lactis which produces the bacteriocin and which thus also carries a resistance gene, may be provided with such multiple copies of the vector; this will thus be able to proliferate without premature destruction by the bacteriocin.

The new bacteriocin may also be prepared by chemical synthesis, for example using solid phase synthesis, advantageously using a polypeptide synthesis apparatus, as commercially available. In such a synthesis, active side chain groupings (e.g. amino or carboxyl groups) of the respective amino acids will be protected and the final step will be deprotection and/or removal from the inert support to which the polypeptide is attached during synthesis.

In building up the peptide chains, one can in principle start either at the C-terminal or the N-terminal although only the C-terminal starting procedure is in common use.

Thus, one can start at the C-terminal by reaction of a suitable derivative of, for example histidine with a suitable protected derivative of leucine. The histidine derivative will have a free α-amino group while the other reactant will have either a free or activated carboxyl group and a protected amino group. After coupling, the intermediate may be purified for example by chromatography, and then selectively N-deprotected to permit addition of a further N-protected and free or activated amino acid residue. This procedure is continued until the required amino acid sequence is

completed.

Carboxylic acid activating substituents which may, for example, be employed include symmetrical or mixed anhydrides, or activated esters such as for example p-nitrophenyl ester, 2,4,5,trichlorophenyl- ester, N-hydroxybenzotriazole ester (OBt), N-hydroxy-succinimidylester (OSu) or pentafluorophenylester (OPFP).

The coupling of free amino and carboxyl groups may, for example, be effected using dicyclohexylcarbodi- imide (DCC). Another coupling agent which may, for example, be employed is N-ethoxycarbonyl-2-ethoxy-1,2-dihydro-quinoline (EEDQ).

In general it is convenient to effect the coupling reactions at low temperatures, for example, -20°C up to ambient temperature, conveniently in a suitable solvent system, for example, tetrahydro- furan, dioxan, dimethylformamide, methylene chloride or a mixture of these solvents.

It may be more convenient to carry out the synthesis on a solid phase resin support. Chloro- methylated polystyrene (cross-linked with 1% divinyl benzene) is one useful type of support; in this case the synthesis will start the C-terminal, for example by coupling N-protected histidine to the support.

A number of suitable solid phase techniques are described by Eric Atherton, Christopher J. Logan, and Robert C. Sheppard, J. Chem. Soc. Perkin I, 538-46 (1981); James P. Tam, Foe S. Tjoeng, and R. B, Merrifield J. Am. Chem. Soc. 102, 6117-27 (1980); James P. Tam, Richard D. Dimarchi and R. B. Merrifield Int. J. Peptide Protein Res 16 412-25 (1980); Manfred Mutter and

Dieter Bellof, Helvetica Chimica Acta 67 2009-16 (1984).

A wide choice of protecting groups for amino acids are known and are exemplified in Schröder, E., and Lübke, K., The Peptides, Vols. 1 and 2, Academic Press, New York and London, 1965 and 1966; Pettit, G.R., Synthetic Peptides, Vols. 1-4, Van Nostrand, Reinhold, New York 1970, 1971, 1975 and 1976; Houben-Weyl, Methoden der Organischen Chemie, Synthese von Peptiden, Band 15, Georg Thieme Verlag Stuttgart, NY, 1983; The Peptides, Analysis, synthesis, biology 1-7, Ed: Erhard Gross, Johannes Meienhofer, Academic Press, NY, San Fransisco, London; Solid phase peptide synthesis 2nd ed., John M. Stewaet, Janis D. Young, Pierce Chemical Company.

Thus, for example amine protecting groups which may be employed include protecting groups which may be employed include protecting groups such as carbobenzoxy (Z-), t-butoxycarbonyl (Boc-), 4-methoxy-2,3,6-trimethyl-benzene sulphonyl (Mtr-), and 9-fluorenylmethoxycarbonyl (Fmoc-). It will be appreciated that when the peptide is built up from the C-terminal end, an amine protecting group will be present on the α-amino group of each new residue added and will need to be removed selectively prior to the next coupling step. One particularly useful group for such temporary amine protection is the Fmoc group which can be removed selectively by treatment with piperidine in an organic solvent.

Carboxyl protecting groups which may, for example be employed include readily cleaved ester groups such as benzyl (-OBZl), p-nitrobenzyl (-ONB), or t-butyl (-tOBu) as well as the coupling on solid supports, for example methyl groups linked to polystyrene.

It will be appreciated that a wide range of other such groups exists as, for example, detailed in the

above-mentioned literature references, and the use of all such groups in the hereinbefore described processes fall within the scope of the present invention.

A wide range of procedures exists for removing amine- and carboxyl-protecting groups. These must, however, be consistent with the synthetic strategy employed. The side chain protecting groups must be stable to the conditions used to remove the temporary α-amino protecting groups prior to the next coupling step.

Amine protecting groups such as Boc and carboxyl protecting groups such as tOBu may be removed simultaneously by acid treatment, for example with trifluoro acetic acid. In building up the peptide chains, one can in principle start either at the C-terminal or the N-terminal although only the C-terminal starting procedure is in common use.

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The following Example is given by way of illustration only:

Example 1

Bacterial strains, media, plasmids, and enzymes. The bacterial strains, plasmids and phases used are listed in Table 1. All lactococcal strains were grown in M17 broth (44) and maintained as frozen stocks at -80°C in M17 broth containing 10% glycerol. Escherichia coli DH5α was used for propagating pUC18 and its derivatives. M13 vectors and clones were propagated in 2x YT (2a) with E. coli JM101 as the host.

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and DNA molecular weight standards were purchased from Bethesda Research Laboratories, Inc. (Gaitherburg, Md.). Calf intestinal alkaline phosphatase, sequence-grade trypsin, and endoprotease glu-C were purchased from Boehringer GmbH (Mannheim, Germany). Sequenase was obtained from United States Biochemical Corp. (Cleveland, Ohio).

Plasmid curing. L. lactis subsp. cremoris LMG 2130 was grown in M17 broth supplemented with 1% glucose at 38°C in the presence of 0.1 μg of novobiocin per ml. Diluted aliquots from this culture were spread on M17 broth-1% glucose plates and incubated at 30°C. Colonies were

scored for bacteriocin production.

Bacteriocin assays. Three methods were used to determine bacteriocin activity. (i) Colonies of possible bacteriocin-producing bacteria were grown on agar plates overnight. A lawn of 3 ml of M17 soft agar (0.7%) containing 100 µl of a fresh culture of the indicator organism was poured over a plate. After incubation overnight at 30°C, the colonies were examined for zones of growth inhibition. (ii) In M17 agar plates, wells with a diameter of 4 mm were made and filled with bacteriocin solutions. After the liquid had been completely absorbed by the gel. M17 soft agar containing the indicator organism was overlaid on the plates to demonstrate bacteriocin activity as described above. (iii) Bacteriocin activity was quantified as described by Geis et al. (15), except that microtiter plates with wells containing 200 µl of M17 broth were used. One unit of bacteriocin activity (BU) was arbitrarily defined as the amount of bacteriocin required to produce 50% growth inhibition (50% of the turbidity of the control without bacteriocin) of L. lactis subsp. cremoris IMN C18 in this assay.

Table 1 : Strains, plasmids, and phages used in this study

Strains, plasmids or phages	Relevant phenotype	Source of reference
Strains		
L.lactis subsp. cremoris		
LMG 2130	LCN-A-producing strain	G. Vegarud
LMG 2131	lcnA derivative of LMG 2130	This study
IMN C18		D. Lillehaug
BC 101		51
L.lactis subsp. lactis		
NIZO 4.25	biovar diacetylactis	J. Narvhaus
IL 1403		6
IMN C18	2130	D. Lillehaug
BC 101		5
E. Coli		
DH5α		17
JM101		31
Plasmids		
pIL253		41
pUC18		53
M13mp18		31
M13mp19		31
pON1	pUC18 with 4-kb HindIII fragment containing lcnA	This work
pON2	pUC18::pIL253 with 4-kb HindIII fragment containing lcnA	This work
pON7	pUC18::pIL253 with 1.2-kb RsaI-HindIII fragment containing lcnA	This work

Purification of LCN-A. The bacteriocin was purified from 1-liter cultures of L. lactis subsp. cremoris LMG 2130. The various steps of the purification procedure were carried out at 4°C unless otherwise stated. The cells were grown to the early stationary phase, and the bacteria were removed by centrifugation at 10,000 x g

for 10 min. The bacteriocin was precipitated from the culture supernatant by the addition of 280 g of ammonium sulfate per liter. Following centrifugation at 10,000 x g for 30 min, the pellet was dissolved in water and adjusted to pH 7.3 by the addition of 0.5 M Na_2HPO_4. This solution was applied to a 10-ml CM-Sepharose column (Pharmacia Uppsala, Sweden) equilibrated with mM sodium phosphate (pH 7.3). The column was washed with 40 ml of 20 mM sodium phosphate (pH 7.3) before the bacteriocin was eluted with 20 ml of the same buffer containing o.3 M NaCl. The bacteriocin was subjected to reverse-phase liquid chromatography at room temperature with fast protein liquid chromatography equipment (Pharmacia). The eluate from the cation exchanger was applied to a 1-ml Phenyl-Superose column (Pharmacia) equilibrated with 10 mM sodium phosphate (pH 7.3). Following washing with 10 mM sodium phosphate (pH 7.3), elution was carried out with a linear gradient of 0 to 60% ethanol at a flow rate of 0.3 ml/min. Purified LCN-A was stored in 60% ethanol-2.5 mM sodium phosphate (pH 7.3) at -20°C. Protein concentrations were determined spectrophotometrically at 280 nm.

Amino acid sequencing. An Applied Biosystems (Foster City Calif.) 477A sequencer was used for amino acid sequencing. The phenylthiohydantion-derivatized amino acid residues were determined on-line with an Applied Biosystems 120 phenylthiohydantoin analyzer. The C-terminal part of the sequence was obtained after cleavage of the Asn-Gly bond with hydroxylamine at pH 9 as described by Bornstein and Galian (3).

DNA isolation, analysis and manipulations. Plasmid DNA was isolated from L. lactis as described by Klaenhammer (23). Small-scale preparation of E. coli plasmid DNA was performed with GeneClean (BIO 101, La Jolia, Calif.). Large-scaled isolation of plasmids from E.

coli was performed by the alkaline lysis method described by Maniatis et al. (25). The M13 plus-strand DNA template for sequencing was prepared from infected 1.5 ml cultures as described previously (2a).

Enzymes for DNA manipulations were used in accordance with manufacturer's specifications. Plasmid DNA from strain LMG 2130 used for cloning was purified by CsCl isopycnic centrifugation (33).

Restriction fragments of the desired size for cloning were isolated and purified from 0.7% agarose gels with Gene-Clean.

DNA cloned in E. coli was subcloned in lactococci as follows. pUC18 plasmids with inserts were fused to pIL253 by EcoRI digestion and ligation. The resultant constructs were transformed into E. coli. Clones were obtained by selection for erythromycin (300 μg/ml) and ampicillin (50 μg/ml) resistance. Plasmid DNA extracted from the clones was used to transform lactococci by electroporation as described by Holo and Nes(20).

Transformation of E. coli was performed by the method of Hanahan (17).

Nucleic acid hybridizations and nucleotide sequencing. On the basis of the sequence extending from amino acid 25 in LCN-A, the following 64-fold-degenerated synthetic oligodeoxynucleotide probe was made (with an Applied Bio-systems 381A DNA synthesizer); 3'-ATIGT(T/C)GT(T/C) TG(I/C)TG(T/C)TTICG(I/C)AAICC-5'. Colony hybridization was performed as described by Hanahan and Meselson (18). Southern blots were made by vacuum transfer (2016 Vacugene; Pharmacia) of restriction endonuclease-digested DNA (fractionated on 0.7% agarose gels) to GeneScreen Plus membranes (NEN Research Products,

Dupont, Boston, Mass,) (42), Hybridization with the 26-mer oligodeoxynucleotide was performed as described by Church and Gilbert (7). Nucleotide sequencing by the didoxynucleotide method (37) was carried out on restriction fragments cloned into M13mp18 and M13mp19 [α-^{35}S]dATP (600 Ci/mmol; Amersham International, Amersham, United Kingdom) was used for labelling.

Nucleotide sequence accession number. The nucleotide sequence presented in this article has been assigned EMBL accession number M63675.

Table 2 : Purification of LCN-A

Fraction	Vol (ml)	A_{280}	Total activity (10^5BUs)	Sp act BUs/ml/A_{280}	Purification (fold)	Yield (%)
Culture supernatant	1,000	14.6	15	102.8	1	100
Ammonium sulfate precipitate	100	5.35	13	2,428	23	87
Cation-exchange chromatography	12	0.17	9.6	4.6×10^5	4,485	64
Reverse-phase chromatography	2	0.51	2.4	2.4×10^5	2,281	16

Purification of LCN-A L. lactis subsp. cremoris LMG 2130 was found to produce a bacteriocin constitutively during growth in M17 medium. A procedure for purifying the bacteriocin from the culture supernatant was developed. The purification scheme is shown in Table 2. The protein was about 95% pure, as judged by amino acid sequence analysis. The amino acid sequence of the purified bacteriocin is as shown above. The bacteriocin was found to contain 54 amino acid residues with a calculated molecular weight of 5,778 which has been

confirm by mass spectrography, thus showing the substantial absence of glycosylation or methylation. No significant sequence similarly was found to any protein or putative gene product in the Swiss-Prot or NBRF data bases.

We have named the new bacteriocin LCN-A. The protein is rich in alanine residues (8 of 54) and glycine residues (8 of 54) and contains only three charged amino acid residues. The calculated isoelectric point of the bacteriocin was 9.2. The extinction co-efficient of LCN-A at 280 nm was estimated to be 1.2 z 10^4 cm^{-1} M^{-1} from its content of tryptophan and tyrosine (4). Thus, pure LCN-A had a specific activity of about 4.9 x 10^5 BUs/mg. Assuming that the activity of LCN-A was not reduced during purification, strain LMG 2130 produced about 3 mg of LCN-A per liter. By comparison, L. lactis subsp. cremoris 346 was found to produce 6 mg of diplococcin per liter (10). The pure bacteriocin was not very soluble in water. Upon storage in aqueous buffers at 4°C, the bacteriocin formed an inactive precipitate. Pure LCN-A could, however, be stored longer than 6 months at ~20°C in 60% ethanol containing 2.5 mM sodium phosphate (pH 7.3) without a detectable loss of activity.

Effect of proteases, LCN-A lost its activity when exposed to various proteases, including the highly specific endoprotease glu-C and trypsin. In phosphate buffer (pH 7.8), endroprotease glu-C could cleave the bacteriocin at one site, between amino acid residues 12 (Asp) and 13 (Leu); trypsin could cleave the bacteriocin at the carboxyl side of its two lysing residues (1 and 21).

Inhibitory spectrum and mode of action. By means of the agar diffusion assay, more than 120 strains of L. lactis

subsp. lactis and L. lactis subsp. cremoris were found to be sensitive to purified LCN-A. Sensitive strains were rapidly killed by the bacteriocin. The viable count of an exponentially growing culture of strain IMN C28 dropped from 2 x $10^8$1/ml after 5 min of exposure to 200 ßUs/ml in M17 medium at 30°C.

Table 3 : Sensitivities of some lactococcal strains to LCN-A

Strain*	Sensitivity (ßUS/ml
L.lactis subsp. cremoris	
IMN C18	5
LMG 2141	1,000
NCDO 607	1.3
NCDO 924	1,000
NCDO 1198	0.4
BC 101	50
BC 101(pON2)	5,000
BC 101(pON7)	5,000
L.lactis subsp. lactis	
NCDO 604	30
IL 1403	0.4
IL 1403(pON2)	1,500
IL 1403(pON7)	1,500
NCDO 176 (biovar diacetylactis)	20
L.garvisae NCDO 2155	5,000

Table 3 shows the sensitivities of various lactococcal strains to LCN-A. Wide variations in sensitivity were found. The most sensitive strains tested appeared to

be more sensitive to the bacteriocin when grown in lactic broth (14) than in M17 medium. In lactic broth, 50% growth inhibition of strain NCDO 1198 was observed at a calculated LCN-A concentration of 40 pg/ml, or 7 pM. This amount corresponds to about 400 molecules of LCN-A per CFU in the assay.

Of the strains tested, only two, the bacteriocin producer itself (LMG 2130) and L. lactis subsp. lactis biovar diaceylactis NIZO 4-25, were resistant. This latter strain, however, was not found to produce the bacteriocin. The nisin (L. lactis subsp. lactis NCDO 496 and NCDO 1403) and diplococcin (L. lactis subsp. cremoris NCDO 893)-producing strains tested were all sensitive to LCN-A and were inhibitory to LMG 2130. In addition, the bacteriocin showed weak inhibition of L. garvieae NCDO 2155 (Table 3).

Identification and cloning of the genetic determinant for LCN-A. An oligodeoxynucleotide probe based on the amino acid sequence of LCN-A was used in Southern hybridization analysis to localize the gene. When plasmid DNA from strain LNG 2130 was probed, one signal, corresponding to a 55-kb plasmid, was observed. Strain LMG 2130 was exposed to plasmid curing. One isolate, LMG 2131, which did not produce LCN-A was found both to be deprived of the 55-kb plasmid and to give no signal on a Southern blot. Furthermore, Southern analysis of LMG 130 plasmid DNA digests revealed signals from a 4-kb HindIII fragment a 1.2-kb HindIII-RsaI fragment and a 0.6-kb DraI fragment. The 4-kb fraction of HindIII-digested LMG 2130 plasmid DNA was cloned in E. coli with pUC18 as the vector. Of 1,400 clones, 10 were found to be positive after screening with the oligodeoxy-nucleotide probe. The recombinant plasmid (pON1) from one of these 10 clones was further restricted with DraI and RsaIII. The fragments that hybridized to the probe,

the 4-kb HindIII fragment, the 1.2-kb HindIII-RsaI fragment, and the 0.6-kb DraI fragment were subcloned into M13Mp18 and M13mp19 to yield inserts in both orientations.

Nucleotide sequence of lcnA. The Hind III-Rsa1 fragment was sequenced. The nucleotide sequence of the two consecutive DraI fragments of 625 and 292 nucleotides is shown in Fig. 1. The entire lcnA gene was contained with the 0.6-kb DraI fragment. Computer analysis of the six possible open reading frames (ORFs) revealed long ORFs only on one of the DNA strands. Mature LCN-A of 54 amino acid residues is encoded by the DNA segment from nucleotide positions 316 to 477. The only possible initiation codon was found at nucleotide position 253, implying that LCN-A is synthesized as a 75-amino-acid precursor containing a 21-amino-acid N-terminal extension. The initiation codon is preceded by the possible Shine-Dalgarno sequence 3' AGGAGA 5' (40). Three putative promotor elements, all showing considerable similarity to the E. coli σ^{70} consensus and streptococcal promoters, were found just upstream of this ribosome binding site (RBS) (Fig. 1) (27,35).

Downstream of lcnA a second ORF, ORF2, was found. Assuming that there is a translation start site at the ATG at nucleotide position 495, this ORF encodes a 98-amino-acid polypeptide. A possible RBS sequence, 5' GAGGATTGA 3', occurs 7 nucleotides from the Met codon. Downstream of ORF2, extending from nucleotide positions 803 and 896, at two regions of dyad symmetry, which could form stem-loop structures with ΔG values of -144.8 and ~102.1 kJmol, respectively (45). The uridine content in their distal stems suggests that these structures constitute Rho-independant terminators of the lcnA transcript. No putative terminator or promotor sequences were found between lcnA and ORF2, indicating

that lcnA and ORF2 may constitute an operon.

No DNA sequence in the EMBL data base showed a high degree of DNA homology to the DNA sequence presented here. The best score found was a 57.4% identity to a 122-bp sequence in the data base.

Cloning in L. lactis. The lcnA gene was cloned in L. lactis. The PIL.253::pUC18 constructions carrying the 4-kb HindIII fragment and the 1.2-kb HindIII-RsaI fragment were named pON2 and pON7, respectively. Neither of these two plasmids caused detectable bacteriocin production in L. lactis subsp. cremoris BC 101. However, when present in BC 101, both pON2 and pON7 conferred resistance to LCN-A. With either plasmid, the LCN-A concentration causing 50% growth inhibition increased from 50 to 5,000 BUs/ml (Table 3); this result was not seen with transformants containing the cloning vector alone. Similar results were observed with other strains of L. lactis (data not shown). The only strain tested that showed bacteriocin production after transformation with the lcnA gene was L. lactis subsp. lactis IL 1403. When carrying pON2 or pON7, L. lactis subsp. lactis IL 1403 produced about 60 BUs/ml. By comparison, the LCN-A-producing strain, LMG 2130, produces about 1,500 BUs/ml.

Sensitivity to LCN-A appears to be general among strains of L. lactis. Since this bacteriocin also is highly specific, it may be used for the identification of L. lactis strains. LCN-A is a hydrophobic protein. Its hydrophobic character was demonstrated by its high affinity for phenyl-Superose. This matrix is intended for use in hydrophobic interaction chromatography, and most proteins bind to it only at high salt concentrations. LCN-A bound to the column in the absence of salt and could only be eluted as an active

bacteriocin by solvents less polar than water.

The toxic effects of nisin have been ascribed to its ability to form pores in cytoplasmic membranes (36). The hydrophobic character of LCN-A suggests that the cytoplasmic membrane may also be the target for this bacteriocin. Calculations made as described by Rao and Argos (34) predicted that the stretch from amino acids 30 to 52 in LCN-A can form a membrane-spanning helix (data not shown). The idea that LCN-A acts on the membrane is further supported by the finding that this bacteriocin causes leakage of intracellular components even in hypertonic sucrose-containing media (unpublished data).

Secreted proteins are usually synthesized as precursors with a short N-terminal extension called the signal peptide, which promotes secretion and which is removed by specific enzymes (1, 43, 49, 50, 52). Comparison of the gene-derived sequence for mature LCN-A with the direct amino acid sequencing data shows that the LCN-A is synthesized as a 75-amino-acid precursor. The LCN-A leader peptide of 21 amino acids has a positively charged N-terminus followed by a hydrophobic stretch typical of signal peptides of gram-positive bacteria (1). Mature LCN-A has a lysine as its N-terminal amino acid. The sequence Ala-Asn-Gly-Gly precedes this lysine in the LCN-A precursor. According to the "-3, -1" rule of Von Heijne (49,50), a signal peptidase could cleave the precursor between the two glycines (-2,-1) but not between the glycine and the lysine (-1, +1). This theory may suggest a stepwise processing of the LCN-A precursor in which a 20-amino-acid peptide and then a glycine are removed from the N terminus to yield mature LCN-A of 54 amino acids.

Three putative promoter elements were found upstream of

the lcnA gene (Fig. 1). Conceivably, transcription initiation could occur 5 to 9 nucleotides downstream of any of the putative Pribnow boxes, yielding leaders of 17 to 33 nucleotides. Overlapping the -10 regions of the putative promoter elements is an inverted repeat sequence that could form a stem-loop structure (Fig. 1). This structure, with a calculated ΔG value of -9.6 kcal/mol (- 40.2 kJ/mol) (45), could represent a Rho-dependent terminator of ORF1.

Strain LMG 2131, which had lost the lcnA gene, was sensitive to LCN-A. This result suggests that the producing organism harbors a gene(s) encoding immunity to the bacteriocin. Strain IL 1403 carrying recombinant plasmid pON7 produced LCN-A and was (by necessity) resistant to the bacteriocin. Thus, the 1.2-kb (RsaI-HindIII fragment appears to carry not only the gene encoding LCN-A but also a genetic determinant for resistance. The DNA sequence of this fragment shows only one complete ORF in addition to the lcnA gene. this is ORF2, located downstream of and in the same operon as lcnA. Hence, the apparently cotranscribed ORF2 is the likely candidate to encode an LCN-A immunity function. A very similar organization of bacteriocin genes and their corresponding immunity genes has been shown for several E. coli bacteriocins (2, 26). ORF2 with Met at nucleotide position 495, preceded by the possible RBS sequence 5' GGATTAG 3', encodes a hypothetical polypeptide of 98 amino acids. Altenatively, there could be an ORF2, with Leu at nucleotide position 540, preceded by the possible RBS sequence 5' AAGAAG 3', with the capacity to encode a hypthetical 83-amino-acid polypeptide. However, codon usage in the 15 N-terminal amino acids of the 98-amino-acid polypeptides correlates well with the compiled codon usage pattern of the rest of the ORF2 polypeptide and of LCN-A, indicating that the ORF2 encodes a 98-

amino-acid polypeptide. Its six N-terminal residues (Met-Lys-Lys-Gln-Ile) show great similarity to signal peptides of gram-positive bacteria. Despite the presence of Glu in positions 7, 9 and 11, the putative signal sequence retains a hydrophobic character extending from amino acid positions 5 to 20. According to the -3, -1 rule of Von Heijne, there is a possible signal peptidase cleavage site after Ala-Thr-Ala at amino acid position 20. Of the 14 gram-positive signal sequences compiled by Abrahamsén et al. (1), 7 contained Ala-X-Ala at their cleavage sites. Moreover, Ala-Thr-Ala was found to be the -3, -1 amino acid sequence of the signal peptidase cleavage site of Bacillus subtilis ß-glucanase (29), possibly suggesting a mature ORF2 protein of 79 amino acids. It remains to be shown whether the ORF2-encoded polypeptide is secreted or anchored within the membrane.

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Claims

1. A polypeptide having or including the amino acid sequence:

1-Lys Leu Thr Phe Ile Gln Ser Thr Ala Ala Gly Asp Leu Tyr Tyr

16-Asn Thr Asn Thr His Lys Tyr Val Tyr Gln Gln Thr Gln Asn Ala

31-Phe Gly Ala Ala Ala Asn Thr Ile Val Asn Gly Trp Met Gly Gly

46-Ala Ala Gly Gly Phe Gly Leu His His

and derivatives and fragments thereof having bacteriocin activity.

2. A polypeptide having or including the amino acid sequence:

Glu Lys Asp Ile Ser Gln Glu Glu Arg Asn Ala Lai Asn Ile Ala Glu

Lys Ala Lai Asp Asn Ser Glu Tyr Lai Pro Lys Ile Ile Leu Asn Leu Arg Lip Ala Leu

Thr Pro Leu Ala Ile Asn Arg Thr Leu Asn Ths Asp Leu Ser Glu Leu Tyr Lys Phe Ile

Thr Ser Ser Lys Ala Ser Ans Lys Asn Leu Gly Gly Gly Lei Ile Met Ser Trp Gly Arg Leu Phe

and derivatives and fragments thereof having bacteriocin immunity activity.

3. A starter culture of microorganisms for use in a microbiological process comprising a polypeptide as claimed in claim 1, said microorganisms being resistant to said polypeptide.

4. A starter culture as claimed in claim 1 in which the microorganisms are lactic acid bacteria or yeasts.

5. A method of cheese or yoghurt production in which a polypeptide as claimed in claim 1 is added to effect lysis of lactic acid bacteria.

6. A method of fermentation for production of ethanol wherein a polypeptide as claimed in claim 1 is used to kill selectively contaminating strains of lactic acid bacteria.

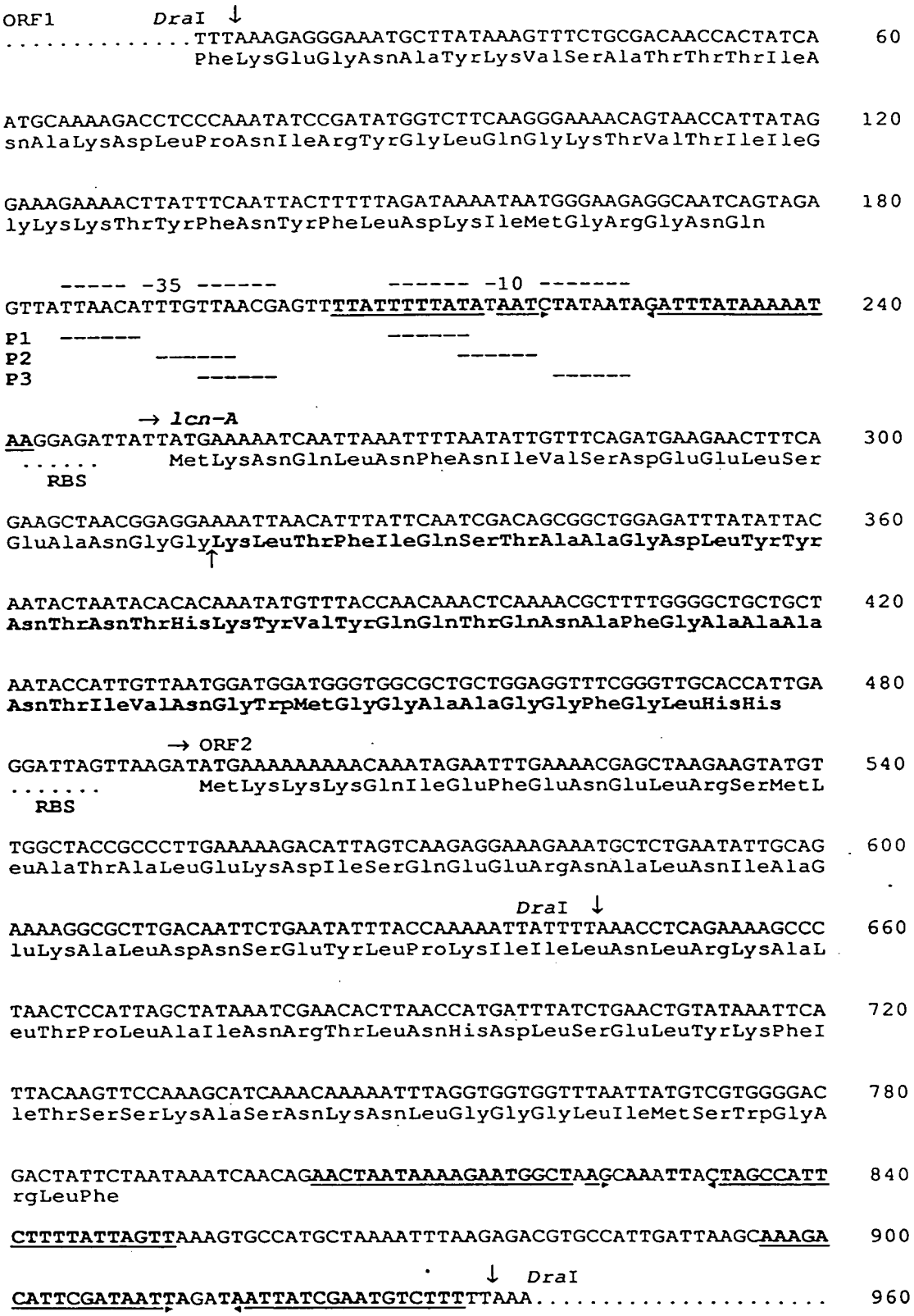
7. A method of isolation of a polypeptide as claimed in claim 1 wherein a culture of a microorganism expressing said polypeptide is subjected to fractionation whereby fractions enriched in said polypeptide are collected.

8. A method as claimed in claim 7 in which the microorganism is Lactococcus lactis subsp. cremoris.

9. A DNA sequence coding for a polypeptide as claimed in claim 1 and/or claim 2.

10. Strains of L. lactis transformed with a vector containing DNA coding for a polypeptide as claimed in claim 1 and/or claim 2.

11. A process for the preparation of a polypeptide as claimed in claim 1 or claim 2 in which a corresponding protected or immobilised polypeptide is subjected to deprotection or removal from an inert support.


ORF1
DraI
TTTAAAGAGGGAAATGCTTATAAAGTTTCTGCGACAACCACTATCA 60
PheLysGluGlyAsnAlaTyrLysValSerAlaThrThrThrIleA
ATGCAAAAGACCTCCCAAATATCCGATATGGTCTTCAAGGGAAAACAGTAACCATTATAG 120
snAlaLysAspLeuProAsnIleArgTyrGlyLeuGlnGlyLysThrValThrIleIleG
GAAAGAAAACTTATTTCAATTACTTTTTAGATAAAATAATGGGAAGAGGCAATCAGTAGA 180
lyLysLysThrTyrPheAsnTyrPheLeuAspLysIleMetGlyArgGlyAsnGln
----- -35 ------ ------ -10 -------
GTTATTAACATTTGTTAACGAGTTTTATTTTTATATAATCTATAATAGATTTATAAAAAT 240
P1 ------ ------
P2 ------ ------
P3 ------ ------
→ lcn-A
AAGGAGATTATTATGAAAAATCAATTAAATTTTAATATTGTTTCAGATGAAGAACTTTCA 300
MetLysAsnGlnLeuAsnPheAsnIleValSerAspGluGluLeuSer
RBS
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GluAlaAsnGlyGlyLysLeuThrPheIleGlnSerThrAlaAlaGlyAspLeuTyrTyr
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AsnThrAsnThrHisLysTyrValTyrGlnGlnThrGlnAsnAlaPheGlyAlaAlaAla
AATACCATTGTTAATGGATGGATGGGTGGCGCTGCTGGAGGTTTCGGGTTGCACCATTGA 480
AsnThrIleValAsnGlyTrpMetGlyGlyAlaAlaGlyGlyPheGlyLeuHisHis
→ ORF2
GGATTAGTTAAGATATGAAAAAAAAACAAATAGAATTTGAAAACGAGCTAAGAAGTATGT 540
MetLysLysLysGlnIleGluPheGluAsnGluLeuArgSerMetL
RBS
TGGCTACCGCCCTTGAAAAAGACATTAGTCAAGAGGAAAGAAATGCTCTGAATATTGCAG 600
euAlaThrAlaLeuGluLysAspIleSerGlnGluGluArgAsnAlaLeuAsnIleAlaG
DraI
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GACTATTCTAATAAATCAACAGAACTAATAAAAGAATGGCTAAGCAAATTACTAGCCATT 840
rgLeuPhe
CTTTTATTAGTTAAAGTGCCATGCTAAAATTTAAGAGACGTGCCATTGATTAAGCAAAGA 900
DraI
CATTCGATAATTAGATAATTATCGAATGTCTTTTTAAA..... 960

Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/01109

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/31 ; C12P7/06 ; A23C19/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12P ; A23C

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 55, no. 5, May 1989, AMERICAN SOCIETY FOR MICROBIOLOGY pages 1187 - 1191; VAN BELKUM, M.J. ET AL.: 'Cloning of two bacteriocin genes from a lactococcal bacteriocin plasmid ' cited in the application see the whole document, especially figure 5 ---	9-10
A	CHEMICAL ABSTRACTS, vol. 112, no. 11, March 12, 1990, Columbus, Ohio, US; abstract no. 95145U, MOERTVEDT, C. & NES, F. 'Bacteriocin produced by a Lactobacillus strain isolated from fermented meat ' page 425 ; see abstract --- -/--	1-14

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 AUGUST 1991	11. 09. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 57, no. 2, February 1991, AMERICAN SOCIETY FOR MICROBIOLOGY pages 492 - 498; VAN BELKUM, M.J. ET AL.: 'Organization and nucleotide sequences of two lactococcal bacteriocin operons ' cited in the application see figures 2,6 ---	1-14
T	JOURNAL OF BACTERIOLOGY vol. 173, no. 12, June 1991, AMERICAN SOCIETY FOR MICROBIOLOGY pages 3879 - 3887; HOLO, H. ET AL.: 'Lactococcin A, a new bacteriocin from Lactococcus lactis subsp. cremoris: isolation and characterization of the protein and its gene ' see the whole document ---	1-14